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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 3/18, C12N 15/62 C12P 21/06 — A1

(11) International Publication Number: WO 91/11454

(43) International Publication Date: 8 August 1991 (08.08.91)

(21) International Application Number: PCT/US91/00040

(22) International Filing Date: 11 January 1991 (11.01.91)

(30) Priority data:
468,724 24 January 1990 (24.01.90) US

(60) Parent Application or Grant
(63) Related by Continuation
US 468,724 (CON)
Filed on 24 January 1990 (24.01.90)

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(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF PURIFYING RECOMBINANT POLYPEPTIDES

(57) Abstract

A method of purifying a desired polypeptide and a kit for practicing the same are disclosed. The method comprises producing a fusion protein comprising a desired polypeptide linked to an affinity peptide that contains an endopeptidase cleavage site. An immobilized endopeptidase is used to separate fusion proteins from impurities and cleave the fusion protein into its substituent parts.

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METHOD OF PURIFYING RECOMBINANT POLYPEPTIDES

FIELD OF THE INVENTION

The present invention relates to a method of obtaining a purified desired protein product by producing it as a recombinant chimeric polypeptides which can be produced in a host, purified and cleaved into constitutive polypeptides.

BACKGROUND OF THE INVENTION

The rapid developments in recombinant DNA methodology have allowed the production of polypeptides, proteins, and their analogs in unlimited quantities in a very short period of time. These developments have created a need to handle purification of these proteins from complex mixtures in highly efficient and predictable manners.

Recombinant DNA technology may be used for the production of desired polypeptides and proteins in host cells. Genes for desired proteins may be isolated from the genetic material of cells which contain the gene in nature. The isolated gene may be inserted and expressed in host cell systems which produce protein products at high levels.

The desired protein products must then be isolated and recovered from the total amount of protein produced by the host cells. The purification of heterologous polypeptides produced by host cells can be very expensive and can cause denaturation of the protein product itself. An overview of protein purification techniques is provided in the Background Art section of U.S. Patent Number 4,782,137 issued Nov. 1, 1988 to Hopp et al. Among the methods to purify proteins that are described in Hopp, the most commonly practiced are ion-exchange, hydrophobic chromatography, and gel filtration. The major disadvantage of these approaches are the lack of specificity of each technique. Thus, these techniques are unsuitable to achieve pure protein in high yields. Even small changes in amino acid composition may change the purification properties. A modified purification procedure needs to be developed and optimized for each new protein. In the case of rDNA derived proteins, structural and functional consequences of heterologous gene expression (H. Bialy, Bio/technology, 5:884, 1987) are additional factors that may make it impossible to predict selection of these purification methods for a given protein.

Desired protein molecules may be isolated from complex mixtures by methods based on solubility differences. For example, isoelectric precipitation makes use of the alteration in protein solubility as a function of pH while fractionation with a solvent is

based on the variation in protein solubility as a function of dielectric constant. Neutral salts, for example, ammonium sulfate, are employed to precipitate proteins due to decreased protein solubility based on high ionic strength of the salt. The drawback is that solvent fractionation can cause protein denaturation. Neither of these methods are capable of purifying proteins beyond a moderate level.

To avoid the negative elements of above techniques, affinity chromatography is often preferred. It is based on the ability of proteins to bind noncovalently but specifically with an immobilized ligand. When used alone, it can purify proteins from complex mixtures without significant loss. It requires the availability of the corresponding ligand for the desired protein. For example, an inhibitor for an enzyme or an antibody for a protein antigen. It should be stressed that in some cases it may be difficult to obtain a specific ligand and such ligands do not exist for all proteins. As a result, this technique has not been applied as a universal method for protein purification.

To circumvent this limitation, recombinant DNA technology may be used to provide an affinity purification system where a linker peptide may be used as an immunoaffinity ligand. This should provide a method that is capable of purifying recombinant proteins in a one-step, using affinity chromatography, without sacrificing high yields. Hopp relates to synthesis of a fusion peptide containing an antigenic linker peptide. The fusion peptide of Hopp is passed through a column containing immobilized antibodies which bind to the antigenic linker. Thus, the fusion peptide may be isolated. The major drawbacks of this technique are either the buffer conditions which are necessary to allow immunogenic complexing or the buffer conditions which must be present to terminate such complexes may denature the desired peptide product. Furthermore, the antigenic linker on the fusion peptide must be removed; an extra step involving addition of a protease and further purification. Finally, the cleavage by the endopeptidase is dependent upon the N-terminal of the desired protein.

Another problem in the area of recombinant proteins is the assay needed to determine the presence of the desired protein. Many proteins and peptides are not enzymes and, thus, their presence may only be determined by in vitro or in vivo biological methods. Purification strategies require a large number of highly accurate data and, therefore, bioassays are time consuming and tend to be inaccurate since a human protein may not work as well in animal models. Although immunoassays can be devel-

oped, this approach requires obtaining antisera for each protein. In addition, due to high specificity of antibodies, a small modification in the protein of interest may alter antibody specificity and, thus, reduce accuracy of the method.

The present invention is directed at the purification of biologically active recombinant polypeptides and/or proteins from bacterial or non-bacterial sources, most preferably those recombinant proteins expressed in a soluble form or secreted from the host as a fusion protein with an affinity peptide. According to the present invention, the desired protein is first produced as a fusion protein which, in addition to the amino acid sequence of the desired protein, contains a linker peptide. The linker peptide of the present invention contains a cleavage site recognized by an endopeptidase and is independent of the N-terminal sequence of the desired protein. When the fusion protein is produced, the desired protein may be isolated and purified by passing the fusion protein through a column containing immobilized endopeptidase. The fusion protein binds to the immobilized endopeptidase for a sufficient amount of time to allow it to be separated from other materials. Furthermore, the immobilized endopeptidase cleaves off the linker peptide, thereby producing pure desired protein.

INFORMATION DISCLOSURE

EP 0 163 573 discloses DNA sequences encoding peptides cleavable by renin and for the insertion of said sequences into plasmid vectors for the production of cleavable fusion proteins. This document discloses the use of a DNA sequence for a renin cleavable linker to connect genes for two polypeptides to form a chimeric gene which encodes a fusion protein. When expressed, the fusion protein may be separated into the substituent polypeptides using renin. This document, however, neither teaches nor suggests the use of the renin cleavable linker in a one step purification and isolation method using immobilized renin columns to purify fusion peptides from crude supernatent and isolate the desired substituent polypeptide from the fusion protein.

European patent application number EP 244 147 relates to the purification of a desired protein by first producing it as a hybrid protein comprising a desired protein portion and a β-galactosidase moiety (BGM) portion linked by a renin cleavage site. The hybrid protein is immobilized in a column containing an affinity linker which recognizes and complexes with the BGM portion. Renin is added to the column, cleaving the hybrid and thereby liberating the desired protein from the column.

U.S. Patent No. 4,782,137 issued November 1, 1988 to Hopp et al., discloses

the synthesis of a fusion protein having a highly antigenic N-terminal portion and a desired polypeptide at the C-terminal portion. According to Hopp et al., the fusion proteins are purified from crude supernatent by passing crude supernatent through a column containing immobilized antibodies which recognize the antigenic portion of the fusion protein. The immobilized antibodies immobilize the protein in the column while the undesired components of the supernatent are eluted. The column conditions can then be changed to eliminate the affinity conditions and cause the antigen-antibody complex to dissociate. The fusion protein is then eluted and collected. The antigenic N-terminal portion must then be removed from the C-terminal portion containing the desirable polypeptide to achieve a purified isolated desired polypeptide. In addition to the necessary second step, another drawback to Hopp et al. is that the conditions which allow for the affinity complexing and/or those which eliminate antibody-antigen recognition may cause denaturation of the fusion protein and loss of bioactivity of the desired protein.

U.S. Patent No. 4,751,180 issued June 14, 1988 to Cousens et al., discloses methods of producing a desired protein product by expression of a fusion gene. Cousens et al. disclose expression of a desired gene linked with a gene which encodes a protein product normally produced in a very large amount in the host. The two genes are linked with the DNA sequence which encodes a cleavable amino acid sequence. Thus, after the fusion protein is produced and purified, the desired protein may be separated from other protein at the cleavable linker.

Haffey, M.L. et al., DNA, Volume 6, 6:565-571 (1987), disclose a synthetic oligonucleotide that codes for an amino acid sequence specifically recognized and cleaved by renin. Haffey et al. teach that oligonucleotide may be inserted into a plasmid expression vector between two genes which encode desired proteins. Expression of the two genes linked with the oligonucleotide results in a fusion peptide which may be cleaved by renin. This publication corresponds to EP 0 163 573 described above.

Hopp, T.P. et al., Bio/Technol. 6:1204-1210, October 1988, disclose addition of an eight amino acid peptide to the N-terminus of a desired recombinant lymphokine in order to provide a antigenic N-terminus which can be used in immunoaffinity purification. This publication corresponds to U.S. Patent No. 4,782,137 described above.

European Patent Application Publication No. 0 244 147 relates to purification

processes of hybrid proteins. A hybrid protein containing β-galactosidase moiety linked to a desired protein through a renin cleavage site is disclosed. The hybrid protein is purified from impurities using affinity chromatography. The affinity matrix binds to the β-galactosidase moiety. The desired protein is released from the hybrid protein by addition of renin to the matrix whereby the desired protein is freed and the β-galactosidase moiety remains bound to the affinity matrix.

SUMMARY OF THE INVENTION

The present invention provides a method of purifying a desired polypeptide comprising the steps of constructing a chimeric gene comprising a gene which encodes a desired polypeptide and which is fused with a DNA sequence that encodes an amino acid sequence cleavable by an endopeptidase, transforming suitable host cells with the chimeric gene in an expression system, producing the fusion polypeptide encoded by the chimeric gene; harvesting polypeptide products produced by the transformed cells, passing the products through a column containing immobilized endopeptidase and collecting desired polypeptide. Furthermore, the present invention provides a kit useful for purifying a desired polypeptide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the purification of biologically active recombinant proteins produced by transformed host cells. The desired biologically active recombinant proteins are most preferably produced in a soluble form or secreted from the host. According to the present invention, the desired biologically active protein is expressed as a fusion protein containing an affinity peptide that can be recognized by an enzyme capable of cleaving the protein at the recognition site. The fusion protein is immobilized and purified from the material present in the secretion media or extraction solution it is contained in and then processed to remove the affinity peptide portion molecule from the portion which comprises the desired protein. According to the present invention, a column is provided which contains an immobilized enzyme which recognizes immobilizes and cleaves the affinity peptide. Thus, the purification and processing steps take place in a single column wherein the impurities are eluted while the recombinant fusion protein is processed into the desired product.

The desired proteins which are produced by recombinant DNA technology and purified are expressed as a fusion protein. In addition to the desired protein, the fusion protein contains the affinity peptide, an amino acid sequence which is recognized by the

immobilized enzyme as a cleavage site. The fusion protein is produced by host cells transformed with the genetic information encoding the fusion protein. The host cells may secrete the fusion protein into the culture media or store it in the cells whereby the cells must be collected and disrupted in order to extract the product.

The culture media containing the secreted fusion protein or the cell extracts containing the fusion protein are passed through the immobilized enzyme-containing column. All of the components of either solution freely pass through the column except the fusion protein. The immobilized enzyme recognizes the affinity peptide and impedes the movement of the fusion protein through the column. Thus, all the impurities are eluted through the column except the fusion protein. The immobilized enzyme cleaves the fusion protein at the scissile bond. This reaction requires a sufficient amount of time to allow the elution of all other components from the original solution. After the cleavage reaction occurs, the fusion protein is released as two components - the desired protein and the cleavage site-containing affinity peptide - which can be collected as a pure eluant. This pure eluant may be separated into its two components using simple techniques well known to those having ordinary skill in the art.

The present invention contains two components: a column containing an immobilized enzyme and a biologically active polypeptide or protein produced as a fusion protein containing an affinity peptide in a suitable host.

The terms "fusion protein" and "chimeric protein" as used herein are interchangeable and refer to polypeptides and proteins which consist of one or two linker peptides with affinity for enzymes which recognize cleavage sites on the linker and a biologically active polypeptide or protein or a short peptide linked directly or indirectly to the linker peptides.

The term "enzyme" referred to herein in the context of an immobilized enzyme and enzyme-containing column, means a polypeptide or protein which recognizes a specific amino acid sequence in a polypeptide and cleaves the polypeptide at the scissile bond. In the preferred embodiment of the present invention, human renin is the enzyme which is used in the immobilized enzyme column.

The term "human renin" referred to herein can be naturally occurring human renin or human renin produced from naturally occurring prorenin by activation, or recombinant human renin, or recombinant human renin obtained by activation of recombinant human prorenin. The recombinant human renin produced from the

activation of recombinant human prorenin is the preferred human renin for immobilization of the fusion protein according to the present invention.

The terms "desired polypeptide" and "desired protein" as used herein are interchangeable and refer to the polypeptide obtained after cleavage by the immobilized enzyme at the scissile bond.

The terms "linker peptide" or "affinity peptide" as used herein are interchangeable and refer to the amino acid sequence which is recognized and cleaved by the immobilized enzyme at the scissile bond.

The term "scissile bond" referred to herein is the juncture where cleavage occurs; for example the scissile bond recognized by human renin may be the Leu-Leu bond or the Leu-Val bond in the linker peptide or affinity pepfide.

The present invention may be used to purify any prokaryotic or eukaryotic protein that can be expressed as the product of recombinant DNA technology in a transformed host cell. These recombinant protein products include hormones, receptors, enzymes, storage proteins, blood proteins, mutant proteins produced by protein engineering techniques, or synthetic proteins.

The chimeric proteins of this invention are prepared by recombinant DNA methodology. In accordance with the present invention, a gene sequence encoding a desired protein is isolated, synthesized or otherwise obtained and operably linked to a DNA sequence encoding the linker peptide. The DNA sequence encoding the linker peptide may be isolated from natural sources or synthesized using techniques well known by those having ordinary skill in the art. The hybrid gene containing the gene for a desired protein operably linked to a DNA sequence encoding a linker peptide is referred to as a chimeric gene.

The chimeric gene is inserted into an expression vector which allows for the expression of the desired chimeric protein in a suitable transformed host. The expression vector provides the inserted chimeric gene with the necessary regulatory sequences to control expression in the suitable transformed host.

There are six elements of control expression sequence for proteins which are to be secreted from a host into the medium, while five of these elements apply to chimeric proteins stored intracellularly. These elements in the order they appear in the gene are: a) the promoter region; b) the 5' untranslated region; c) signal sequence; d) the chimeric coding sequence; e) the 3' untranslated region; f) the transcription termination site.

Fusion protein which are not secreted do not contain c), the signal sequence.

Methods and materials for preparing recombinant vectors and transforming host cells using the same, replicating the vectors in host cells and expressing biologically active foreign polypeptides and proteins are described in Principles of Gene Manipulation, by Old and Primrose, 2nd edition, 1981.

In addition to the genetic information necessary to encode and produce the fusion peptide, a column containing an immobilized enzyme is provided. This immobilized enzyme must retain its ability to recognize and cleave the specific amino acid sequence present in the linker peptide.

Additionally, the present invention relates to: genes which encode fusion proteins, expression vectors containing the same, microorganisms transformed with these expression vectors, and a process for obtaining these genes, expression vectors, and microorganisms transformed with said vectors.

In the preferred embodiment of the present invention, the linker peptides with affinity for human renin in accordance with this invention are defined by the general formula:

R1-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser-R2

where R1 is a hydrogen atom, an amino acid, a sequence of few amino acids, a sequence of several amino acids, or a polypeptide. R2 represents a polypeptide or protein of interest.

Other suitable preferred linker peptides with affinity for human renin in accordance with this invention include peptides which comprise the following amino acid sequences:

Pro-Phe-His-Leu-Val-Ile-His-Ser; and,

Pro-Ile-Pro-Phe-His-Leu-Val-Ile-His-Ser.

In the preferred embodiment of the present invention, a column is provided that contains immobilized renin which recognizes and cleaves the above described linker peptides.

According to the present invention, the desired polypeptides produced may include tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

Conventions used to represent plasmids and fragments in Charts below, though unique to this application, are meant to be synonymous with conventional representations of plasmids and their fragments. Unlike the conventional circular figures, the single line

figures on the charts represent both circular and linear double-stranded DNA with initiation or transcription occurring from left to right (5' to 3'). Asterisks (*) represent the bridging of nucleotides to complete the circular form of the plasmids. Fragments do not have asterisk marks because they are linear pieces of double-stranded DNA. Endonuclease restriction sites are indicated below the line. Genes and fragments are identified below the line.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Example 1. IMMOBILIZATION OF RECOMBINANT HUMAN RENIN IN A COLUMN Biotinylation of renin:

In order to be able to immobilize active renin to a matrix, either the renin or the matrix has to be chemically modified to bind the other. In this example, the renin is chemically modified using a series of techniques revolving around the Biotin/Avidin system. That system is used because it is known to be useful in the immobilization of other enzymes while keeping them in an active form. Another reason is that the reagents used in the system are readily available from a number of sources.

Recombinant human renin is first biotinylated two different ways using two different protein modification reagents:

1. NHS-Biotin; and,
2. Sulfosuccimidobiotin.

These reagents are used to biotinylate the recombinant human renin as follows:

The human renin to be modified is first put into solution. Recombinant human renin is dissolved in water at a concentration of 2mg/ml. Reaction samples are prepared by combining .1ml aliquots of renin solution with .9mls of a 1mg/ml BSA solution dissolved in .1M $NaHCO_3$ pH 8.0 and dialyzed into solution of .1M $NaHCO_3$ overnight.

The human renin is modified by Reagent 1 using the following protocol. Reagent 1 is dissolved in DMSO at a concentration of 1mg/ml. A reaction sample of human renin is combined with .12mls of reagent/DMSO solution and reacted for 1hr at ambient temperature.

The human renin is modified by Reagent 2 using the following procedure. Reagent 2 is dissolved in dialysis buffer at a concentration 4.43mg/ml. To a renin sample, .057ml of the Reagent solution is added and reacted for 4hrs at ambient temperature.

Both reaction mixtures are separately then dialyzed extensively into 20mM NaH2PO4, 150mM NaCl, .1% Na Azide, pH 7.4. Modifications 1 and 2 both result in the maintenance of significant amounts of renin. Full activity is essentially maintained. The two modified renins demonstrate no loss in renin activity and allow for the immobilization these forms of renin on a solid matrix.

Immobilization of different biotinylated renins on Strepavidin Sepharose.

The biotinylated forms of recombinant renin produced above are attached to commercially prepared columns containing Strepavidin covalently bound to Sepharose. The interaction between biotin and avidin, although not covalent, has a dissociation constant on the order of 10-15 M. The modified renin is attached to the avidin through this affinity without loss of renin activity. The strepavidin form of avidin is used because it is reported to have lower non-specific binding of proteins than avidin.

The two active derivatives of renin are dialyzed into PBS/TWEEN (20mM NaH2PO4, 150mM NaCl, .2% TWEEN pH 7.5). One ml samples of biotinylated renin were loaded onto separate .5ml columns of strepavidin Sepharose which has been equilibrated in PBS/TWEEN. The columns are then washed with PBS minus TWEEN and fractions which do not bind to the columns are assayed for renin activity using the standard renin activity assay.

In both cases, less than .01% of the modified renin applied to the column does not bind to the column. Thus, the biotinylated forms of renin not only retain renin activity but are capable of binding strepavidin. The renin which bound the strepavidin column following biotinylation bound specifically through the biotin moiety. The chemically modified recombinant human renin is immobilized in a solid support. Two different columns are produced.

Hydrolysis of renin substrate on immobilized renin columns

To show that the renin still has activity even in a bound state, purified human angiotensinogen is passed over the columns and the renin cleavage product is measured in the standard ANG-I RIA. Samples of human renin substrate are dialyzed into 150mM Na2HPO4, 160mM NaCl, 3mM EDTA, pH 6.0 and 2.5mls of a 5μg/ml solution of angiotensinogen is chromatographed on both renin columns by passing the angiotensinogen over the columns three times and collecting the material which does not bind. Columns used are .5mls and equilibrated with the buffer described above. The chromatography is done at ambient temperature under gravity. The columns are then washed

with the starting buffer and fractions are collected. All the fractions are assayed for the presence of ANG-I using the RIA and the total amount of ANG-I collected is compared to the total ANG-I expected. The expected amount of ANG-I is determined by incubating a 50ul aliquot of the pre-column angiotensinogen with an excess amount of renin at 37 C for 2hrs.

The immobilized renin which had been derivatized using either NHS-Biotin or Sulfosuccinimidobiotin is essentially fully active toward renin substrate under the conditions described above. Both of these columns completely cleave substrate, indicating that the renin which had been immobilized is still active and capable of hydrolyzing substrate.

Example 2 CONSTRUCTION OF A FUSION PEPTIDE CONTAINING AN AFFINITY PEPTIDE AND HIV RT

There are basically three known properties of human renin that used in conjunction, for the immobilization of chimerics containing renin-specific handle:

a) Much slower hydrolysis of non-primate substrates at its optimum pH;

b) Slower catalysis when used in an environment with a pH slightly higher than the optimal pH;

c) Retention of activation at 4° C; similar to many other proteins which undergo inactivation at higher temperatures.

Once biologically active human renin has been immobilized it is possible to immobilize recombinant proteins (enzymes, hormones, and receptors) containing a linker peptide at the N-terminal. The catalytic rate of cleavage in the presence of human renin is highly species dependent with respect to substrate used. The N-terminal side of the scissile bond (↑) from many species are identical while differences in specific amino acid residues on the C-terminal side of scissile bond are different and are known to affect hydrolysis of various substrates by human renin. For example, rat plasma angiotensinogen is hydrolyzed by human renin 25-fold slower than human angiotensinogen.

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His- Human

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr- Rat

----------N-terminal----------↑---C-terminal----

Fusion proteins prepared by linking DNA encoding for the linker peptide in frame with a gene for heterologous protein are used for immobilization of fusion proteins with renin columns.

For example:

Pro-Ile-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser-RT;

wherein RT is a desired protein.

Construction of Oligonucleotides Which Are Used to Construct Gene Sequences That Encode Affinity Peptides and HIV RT

The following oligonucleotides (1-6) which are used to construct linker sequences of construct #1 (Pro-Ile-Pro-Phe-His-Leu-Val-Ile-His-Ser-) and construct #2 (Pro-Ile-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser-) are synthesized separately using standard methods.

1. 5' ATT GAG ACT GTA CCA GTA AAA TTA AAG CCA GGA ATG GAT GGC CCA
AAA GTT AAA CAA TGG 3'

2. 5' CCA TTG TTT AAC TTT TGG GCC ATC CAT TCC TGG CTT TAA TTT TAC TGG TAC AGT CTC AAT AGG G 3'

3. 5' AA TTC ATG CCC ATT CCC TTT CAC TTA GTA ATT CAC AGC CCC ATT AGC CCT 3'

4. 5' CT AAT GGG GCT GTG AAT TAC TAA GTG AAA GGG AAT GGG CAT G 3'

5. 5' AA TTC ATG CCC ATT CCC TTT CAC TTA CTA TAC TAC AGC CCC ATT AGC CCT 3'

6 5' CT AAT GGG GCT GTA GTA TAG TAA GTG AAA GGG AAT GGG CAT G 3'

Polyacrylamide gels are used to purify these oligonucleotides. The purified oligonucleotides are annealed and ligated in the following formats.

Construction #1

5'—oligo #3——3' 5'—oligo #1—3'

3'-oligo #4--5' 3'--oligo #2--5'

Construction #2

5'---oligo #5------3' 5'---oligo #1---3'

3'-oligo #6---5' 3'--oligo #2--5'

The above constructions contain the following restriction sites:

5'EcoR1----------3'

3'----------5'Bal I

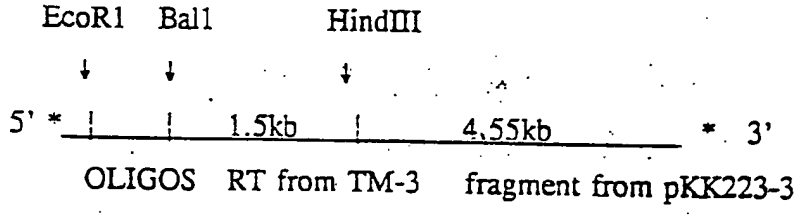
The above EcoR1/Bal I pieces are ligated into the final vector as 1 part of a three-way ligation. The other two pieces for this ligation are obtained as follows.

Construction of DNA Expression Vectors Which Contain Affinity Peptide DNA Sequences Fused To HIV Reverse Transcriptase RT Gene

The gene for HIV reverse transcriptase is widely available and the methods used to insert restriction enzyme sites at its flanking regions are well known to those having ordinary skill in the art. In this example, a clone, TM-3, described by Diebel, M. R. et al, AIDS and Human Retroviruses, In Press (1990), which contains the gene for HIV reverse transcriptase, RT is cut with Bal I/Hind III to release the 1.5Kb HIV RT gene fragment.

Plasmid pKK223-3 (Pharmacia) is cut with Eco R1/Hind III and the 4.55Kb fragment was isolated.

The two desired fragments are purified on agarose gels and mixed with either of the two ligated sets of oligos for construction # 1 and Construction # 2. These are ligated to the final plasmid to yield HIV RT containing the linker at its N-terminal. The structure of a recombinant chimeric (linker-HIV RT) expression vector is shown below.



Production of Fusion Proteins

The plasmids are transformed into the JM-109 strain of E. coli using standard protocols. Cells were grown on ampicillin containing plates. Clones containing reverse transcriptase (RT) activity are selected and sequenced using ^{32}P dATP. Colonies containing the correct DNA sequence are grown for expression of the chimeric genes. For preparation of the chimeric proteins for purification in accordance with the current invention, approximately 3g of E. coli cell paste is suspended in 30mls 0.25M potassium phosphate, pH 7.2 containing 1mM dithiothreitol (DTT), EDTA, phenylmethylsulfonyl fluoride (PMSF), and benzamidine HCL, 10mg/liter aprotinin, leupeptin, and bestatin. This suspension is passed through a French Press three times to break the cells. Cell lysates are centrifuged at 12,000 rpm for 1hr. The HIV RT is detached from the fusion protein and isolated from the crude supernatant using immobilized human renin.

Example 3 CONSTRUCTION OF A FUSION PEPTIDE CONTAINING A MODIFIED ANGIOTENSIN LINKER AND tPA

Construction of Plasmid for Extended (Ser-Tyr), Modified AngI (+3, Val-Ile-His)-tPA

This example uses the sib transcriptional terminator as a regulatory sequence for terminating transcription of the fusion protein gene comprising the tPA gene linked to a modified angiotensin linker. The sib transcription terminator is isolated from bacteriophage lambda. The methods performed to accomplish this are well know to those having ordinary skill in the art using readily available materials (See Weighous, T. F. and W. G. Tarpley, Biochem. and Biophys. Res. Comm., Vol. 143, 2:593-599, 1987).

The Hind III site of pGEM-4 (Promega) is replaced with a Sal 1 linkers (New England Biolabs).

The sib transcriptional terminator is inserted as an Xho fragment into the Sal 1 site of a modified pGEM-4.

A 473bp piece from the 5' end of the tPA gene is obtained as follows. The full length tPA cDNA is cleaved with Hgal, releasing a 517 bp Hgal fragment. Termini are made flush with Klenow and BamH1 linkers are added to the termini using standard procedures. The modified fragment is then digested with BamH1 and Narl to produce a 440 bp BamH1 - Narl fragment representing the 5' portion of the tPA sequence beginning with a BamH1 site at nucleotide number 78 (numbering according to Pennica

et al.). This fragment was subsequently assembled with the 3' portion of the gene represented by a Nar1-BglII fragment isolated from the full length tPA cDNA and the vector pKC7 which is digested with BamH1 and BglII, yielding plasmid pPSA. This plasmid contains a version of the tPA gene which contains an engineered BamH1 site at the 5' end (78) throughout the full 3' non-coding portion of the gene.

The 473bp fragment is then inserted as a Bam H-1/Nar I fragment 3' to the terminator.

The oligonucleotides corresponding to the ANG-I(+3) linker are reconstructed from 4 oligonucleotides; the 29-mer GATCTTACGACATAGTGTACATACACCCC and the 37-mer TTCCACCTCGTCATCCACTCTTACCAAGTGATCTGCA and their complementary oligonucleotides. These oligos are purified, kinased, annealed and re-purified according to standard methods and then inserted as a Bgl II/Pst I fragment into the 5' end of the tPA gene.

The construct is sequenced by the dideoxy method to ensure correct assembly. The remaining 3'-end of the tPA gene is isolated as a Nar I/Bam H1 fragment from ptPA-IPA and ligated to the purified 5' Bam HJ-1/Nar fragment of DNA which contains the oligonucleotides for ANG-I(+3).

The full length tPA gene containing the oligonucleotides is gel purified and inserted as a Bam H1 fragment into the Bgl II site in the TFW-9 vector.

The BPV genome necessary for expression in mammalian cells is then added as a Bam H-1 fragment and the entire construct was used to transfect C-127 cells as described.

Culturing of C-127 Murine Cells

Murine C-127 cells are obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's minimal essential media DMEM (Gibco Cat.#320-1965) containing 10% fetal calf serum and 100 units/ml Penicillin and 100 μg/ml streptomycin. Cells were transfected by standard procedures using 5μg DNA. Three weeks later, transformed foci are picked and expanded as individual cell lines. The individual cell lines are grown and assayed for secreted tPA activity. Cell line DE-9-22 was found to contain the most secreted tPA activity and was chosen for further expansion.

Cells are then grown in the above serum-containing media containing 1 μg/ml aprotinin and 10 mM EACA and expanded to 100 mm plates. These cells are then

passed and seeded on Cytodex 1 microcarrier beads (Pharmacia) in 3,000 ml spinner flasks at a $6.3x10^7$ cells/ml and grew to a density of $1.0x10^9$ cells/ml (after nine days). Once the cells reach maximum density the culturing protocol is modified as follows. Cells are rinsed three times with an isotonic buffer and placed in serum-free media which consists of equal parts DMEM 320-1885 (Gibco) and MCDB Medium 301 Formula 78-0037AJ (Gibco) supplemented with 10 mM Hepes, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite and 4 mM L-glutamate. There may also be some residual serum present in the medium. The media also contains antibiotics and protease inhibitors. The media may be harvested and the components of the fusion protein recovered by passing the lysates through an immobilized renin column.

Example 4 PROCEDURE FOR COLLECTING FUSION PROTEINS USING IMMOBILIZED HUMAN RENIN COLUMN

Lysates from E. coli expressing the recombinant chimerics or conditioned media into which the chimeric has been secreted by mammalian cells, are added to the immobilized renin column at a pH 7.0 which is slightly higher than the optimum pH of human renin. The column is allowed to remain at 5°C. Recombinant proteins to be used as a starting material includes purified products, partially purified proteins obtained during purification process or crude extract containing the desired recombinant protein.

It is recommended that the expressed polypeptide be in soluble form, preferably in the absence of denaturants. The use of a buffer solution compatible with the column equilibration buffer is recommended, to avoid inhibition of immobilized human renin. Binding pH is in the range 5 to 8.5; from the point of view of stability of both immobilized human renin and the desired recombinant protein. A pH range of about 5.5 to 7.5 is especially preferable. Binding time ranges from about 30 minutes to 50 hrs. Binding temperature ranges from 5 C to 37 C, more preferably 5 C for chimerics containing the human Leu-Val scissile bond and 25 C for chimerics containing the rat Leu-Leu scissile bond.

Since immobilized form of human renin is employed for purification of recombinant proteins, the enzyme is used repeatedly and therefore the method is economical and efficient.

As hosts, both E. coli and mammalian cells are preferred hosts. Of these two, E. coli is the preferred host according to this invention.

CLAIMS

1. A method of purifying a desired polypeptide comprising the steps of:

a) constructing a chimeric gene comprising a gene which encodes a desired polypeptide and which is fused with a DNA sequence which encodes an amino acid sequence cleavable by an endopeptidase;

b) transforming suitable host cells with said chimeric gene in an expression system such that said chimeric gene is expressed, producing a fusion polypeptide encoded thereby;

c) harvesting polypeptide products produced by said transformed cells and passing said products through a column containing immobilized endopeptidase; and

d) collecting desired polypeptide.

2. A method according to Claim 1 wherein said endopeptidase is renin.

3. A method according to Claim 2 wherein said amino sequence cleavable by renin comprises an amino acid sequence selected from the group consisting of: Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser;Pro-Ile-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser;and,Pro-Phe-His-Leu-Val-Ile-His-Ser.

4. A method according to Claim 3 wherein said amino sequence cleavable by renin comprises the amino acid sequence: Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser.

5. A method according to Claim 4 wherein said desired polypeptide is selected from the group consisting of tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

6. A method according to Claim 1 wherein said desired polypeptide is selected from the group consisting of tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

7. A method according to Claim 2 wherein said desired polypeptide is selected from the group consisting of tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

8. A method according to Claim 3 wherein said desired polypeptide is selected from the group consisting of tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

9. A kit for purifying a desired polypeptide comprising:

a) a DNA molecule which contains a DNA sequence which encodes an amino acid sequence cleavable by an endopeptidase, said DNA molecule is used to construct a chimeric gene comprising a gene which encodes a desired polypeptide fused with said DNA sequence which encodes an amino acid sequence cleavable by an endopeptidase; and,

b) a column containing immobilized endopeptidase, said column is used to harvest polypeptide products produced by expression of said chimeric gene in a transformed cell by passing said products through a column containing immobilized endopeptidase.

10. A kit according to Claim 9 wherein said endopeptidase is renin.

11. A kit according to Claim 10 wherein said amino sequence cleavable by renin comprises an amino acid sequence selected from the group consisting of: Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser;Pro-Ile-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser;and,Pro-Phe-His-Leu-Val-Ile-His-Ser.

12. A kit according to Claim 11 wherein said amino sequence cleavable by renin comprises the amino acid sequence: Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser.

13. A kit according to Claim 12 wherein said desired polypeptide is selected from the group consisting of tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

14. A kit according to Claim 9 wherein said desired polypeptide is selected from the group consisting of tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

15. A kit according to Claim 10 wherein said desired polypeptide is selected from the group consisting of tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

16. A kit according to Claim 11 wherein said desired polypeptide is selected from the group consisting of tPA, IL-1, BST, IL-1 receptor, CD4, HIV RT and human nerve growth factor.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/00040

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 3/18, C 12 N 15/62, C 12 P 21/06

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	EP, A, 0244147 (MERCK & CO INC.) 4 November 1987 see abstract; claims (cited in the application)	
A	WO, A, 8804692 (IMMUNEX CORP.) 30 June 1988 see page 3, lines 1-34; claims	
A	EP, A, 0157235 (BAYER AG) 9 October 1985 see claims	
A	EP, A, 0327522 (NYGREN et al.) 9 August 1989 see claims; column 4, line 14 - column 5, line 34	

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19th April 1991	11. 06. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. SOTELO

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP, A, 0163573 (MERCK & CO. INC.) 4 December 1985 see claims (cited in the application)	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9100040 SA 43945

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 27/05/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0244147	04-11-87	JP-A- 62259596	11-11-87
WO-A- 8804692	30-06-88	US-A- 4851341	25-07-89
		AU-A- 1056188	15-07-88
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EP-A- 0157235	09-10-85	DE-A- 3410437	26-09-85
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EP-A- 0327522	09-08-89	JP-A- 2005887	10-01-90
		SE-A- 8800378	06-08-89
EP-A- 0163573	04-12-85	JP-A- 60262595	25-12-85